

Coupling between agonist and chloride ionophore sites of the GABA_A receptor: agonist/antagonist efficacy of 4-PIOL

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Abstract

Eight γ -aminobutyric acid (GABA) mimetics were tested on their ability to differentiate native GABA_A receptor subtypes present in various rat brain regions. In rat brain cryostat sections, little regional variations by the agonistic actions of muscimol, thiomuscimol, 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol, piperidine-4-sulphonic acid, taurine and β -alanine on [³⁵S]t-butylbicyclophosphorothionate ([³⁵S]TBPS) binding to GABA_A receptor channels were found. They were very similar to those found for GABA itself and indicated no direct correlation with single subunit distributions for any of these compounds. Only the low-efficacy GABA mimetic 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) acted like a weak partial agonist or antagonist depending on the brain area. As the cerebellar granule cell layer was relatively insensitive to both modes of action, we tested 4-PIOL in recombinant $\alpha 1\beta 2\gamma 2$ (widespread major subtype) and $\alpha 6\beta 2\gamma 2$ (cerebellar granule cell restricted) receptors where it had different effects on GABA-modulated [³⁵S]TBPS binding and on electrophysiological responses. 4-PIOL may thus serve as a potential lead for receptor subtype selective compounds. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

GABA_A receptors are responsible for the fast synaptic inhibitory neurotransmission in the mammalian brain. They belong to the superfamily of ligand-gated ion channels and are characterized by the pentameric structure of the integral anion channel. It is composed of a variable array of polypeptide subunits ($\alpha 1 - 6$, $\beta 1 - 3$, $\gamma 1 - 3$, δ , π , ϵ and θ), all of which are products of separate genes (Barnard et al., 1998; Hedblom and Kirkness, 1997; Bonnert et al., 1999; Sinkkonen et al., 2000). The receptor subunits are differentially expressed in all brain regions, producing a presently unknown number of receptor subtypes. Their structural diversity forms the basis for the functional and pharmacological heterogeneity of GABAergic neurotrans-

mission. The drug therapy of insomnia, anxiety, epilepsy and surgical sedation presently targets most GABA_A receptor subtypes non-selectively, but the heterogeneity of the receptors theoretically holds promise for a brain region- or even cell-selective pharmacological intervention.

Most of the known pharmacological heterogeneity concerns the sensitivity of the benzodiazepine site (Lüddens and Korpi, 1995a). It depends, firstly, on the α variant, with $\alpha 4$ and $\alpha 6$ subunit-containing receptors being practically insensitive to benzodiazepine site agonists (Lüddens et al., 1990; Wisden et al., 1992) and, secondly, on the presence of a γ subunit in $\alpha X\beta X\gamma X$ receptors for the formation and function of the binding site (Pritchett et al., 1989; see Sigel and Buhr, 1997). In addition to receptor subtype-selective interactions via the benzodiazepine site (Lüddens and Korpi, 1995a; Quirk et al., 1996; Skolnick et al., 1997), we have identified novel subunit requirements for non-benzodiazepine site compounds like furosemide, a Na⁺, K⁺, 2Cl⁻ cotransporter blocker [presently the most GABA_A receptor subtype-selective antagonist (Korpi and Lüddens, 1997; Korpi et al., 1995b)] and clozapine, an

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atypical antipsychotic drug [GABA_A receptor antagonistic elsewhere in the brain except in the furosemide-sensitive cerebellar granule cell layer (Korpi et al., 1995a)]. Several other non-benzodiazepine site ligands also show brain regional heterogeneity in their actions (Edgar and Schwartz, 1990; Gee et al., 1988; Sapp et al., 1992), but less clearly than furosemide and clozapine. These interactions have been investigated in autoradiographic and homogenate binding assays using [³⁵S]t-butylbicyclophosphorothionate ([³⁵S]TBPS). [³⁵S]TBPS binds to GABA_A receptors apparently close to the ionophore in an anion-dependent manner (Squires et al., 1983; Jursky et al., 2000) and the assays have been proven as biochemical functional tests being sensitive to all known GABA_A receptor agonists and antagonists. They have been confirmed in electrophysiological recordings, which directly reflect receptor function.

Especially, the observations on furosemide and clozapine indicate the usefulness of the approach. Here we have successfully applied this approach to brain regional diversity of GABA_A receptors at the level of the GABA binding site in order to study the usefulness of various GABA mimetics as new lead compounds for subtype-selective ligands. GABA is known to increase [³⁵S]TBPS binding to $\alpha 1$ subunit-containing receptors at low and decrease it at higher micromolar concentrations (Korpi and L ddens, 1993; L ddens and Korpi, 1995b). As well, it is more potent to inhibit [³⁵S]TBPS binding in the cerebellar granule cell layer than in other brain regions (Korpi et al., 1992). As in this respect, little more is known regarding GABA itself and other GABA_A receptor agonists of various chemical classes, we compared the effect of GABA on [³⁵S]TBPS binding to muscimol, the prototypic GABA_A receptor-selective agonist, to its derivative thiomuscimol (Nielsen et al., 1995) and to β -alanine and taurine, two endogenous amino acids affecting both the glycine and the GABA_A receptors (Horikoshi et al., 1988). In addition, we employed 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) and piperidine-4-sulphonic acid, two potent but structurally diverse derivatives that in benzodiazepine binding assays have shown partial agonist activity (Braestrup et al., 1979; Falch et al., 1985; Karobath and Lippitsch, 1979), and a compound classified as a low-efficacy partial agonist, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) (Krogsgaard-Larsen et al., 1994). Since only the latter compound showed potential selectivity in native receptors, we applied whole-cell patch-clamp recordings on recombinant $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors to allow for its direct functional characterization.

2. Materials and methods

2.1. Animals

Adult, male Wistar rats (350 g, supplied by the Department of Laboratory Animals, University of Helsinki) were

maintained in groups of 3–5 animals in stainless-steel wire-mesh cages with R3 rat pellet food (Ewos, S dert lje, Sweden) and tap water available *ad libitum*. For autoradiography, the animals were decapitated with a guillotine and their whole brains carefully dissected and frozen on dry ice.

2.2. Autoradiography

The procedure used has been described in detail by Korpi et al. (1996). Briefly, 14- μ m frontal sections were cut with a Microm HM 500 OM cryostat (Walldorf, Germany). The frontal sections were at the following levels (in mm) from the bregma according to Paxinos and Watson (1982): 6.7, 3.2, 1.7, –0.8, –1.8, –3.3, –5.3, –6.3, –8.7 and –10.5. Serial sections were preincubated on ice three times for 10 min in 50 mM Tris–HCl (pH 7.4) supplemented with 1 mM EDTA (Merck, Darmstadt, Germany). Incubation of sections with [³⁵S]TBPS (about 200 dpm/ml, adjusted to 6 nM with cold TBPS, DuPont de Nemours, NEN Division, Dreieich, Germany) was performed in 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl using 800- μ l liquid drops over sections on object glasses in a humid chamber for 90 min at room temperature (22 C) either in the presence of 3 μ M GABA (Research Biochemicals, Natick, MA), 1 μ M muscimol (Sigma, St. Louis, MO), 10 μ M thiomuscimol (Research Biochemicals), 30 μ M THIP (Research Biochemicals), 5 mM taurine (Sigma) or 1 mM β -alanine (Sigma). The concentrations of the agonists were based on preliminary assays and produced approximately 50% inhibition of [³⁵S]TBPS binding when averaged over the whole rat brain. In a second experiment, the added agonists were as follows: 2 and 5 μ M GABA in the absence and presence of 10 μ M piperidine-4-sulphonic acid (Research Biochemicals), as well as 10 μ M piperidine-4-sulphonic acid alone. In the third experiment, 2 μ M GABA was used in the absence and presence of 300 μ M 4-PIOL (donated by Povl Krogsgaard-Larsen, Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, Copenhagen, Denmark), which in a preliminary experiment produced maximal inhibition of [³⁵S]TBPS binding. Nonspecific binding was defined in the presence of 20 μ M picrotoxinin (Sigma) and it was at the background level. After incubation, the sections were washed three times for 15 s in ice-cold incubation buffer, dipped into distilled H₂O, dried under a fan at room temperature, and exposed to Hyperfilm Bio Max MR (Sigma) for 7–10 days.

Labeling intensities of various regions of the sections were quantified from the films by using AIS image analysis devices and programs (Imaging Research, St. Catharines, Canada). Locations of various brain areas on exposed films were identified with the aid of the same brain sections stained with thionin. Binding densities for each brain area were averaged from measurements from one to two sections. Plastic ¹⁴C-standards (Amersham,

Buckinghamshire, UK) exposed simultaneously with the brain sections were used as reference with the resulting basal binding values given as radioactivity levels estimated for gray matter areas (nCi/g).

2.3. Recombinant GABA_A receptors and ligand binding assays

Transfection and ligand binding assays with recombinant $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ GABA_A receptors were carried out as described in detail (Korpi and L  ddens, 1993; L  ddens and Korpi, 1995b). Briefly, human embryonic kidney (HEK) 293 cells were transfected using phosphate precipitation method with rat $\alpha 1$, $\alpha 6$, $\beta 2$ and $\gamma 2$ cDNAs in eukaryotic expression vectors. Two days after transfection the cells were harvested, homogenized with a polytrone, washed by centrifugation and stored at -80°C until binding experiments were performed. Washed membranes (about 200 μg protein/ml) were incubated in the presence of [^{35}S]TBPS at 6 nM concentration with and without GABA and 4-PIOL for 90 min in 50 mM Tris–citrate buffer, pH 7.4, supplemented with 200 mM NaCl. The bound fraction was separated by rapid filtration of the membrane homogenates onto glass fiber filters under reduced pressure, the filters dried, immersed in scintillation fluid and counted for radioactivity using a Beckmann liquid scintillation counter. Picrotoxinin at 20 μM was used to define the nonspecific binding that was in $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors 9 ± 3 and 38 ± 8 (m%, mean $\pm$ SD, $n = 3$) of the basal [^{35}S]TBPS binding, respectively. It was subtracted from the basal binding to give the specific binding.

2.4. Electrophysiology

For electrophysiological recording HEK-293 cells were passaged and replated on 12-mm glass coverslips located in 9.6-cm plastic dishes filled with 10 ml of Minimum Essential Medium (MEM, Gibco) supplemented with 158 mg/l sodium bicarbonate, 2 mM glutamine (Gibco), 100 U/ml penicillin–streptomycin (Gibco), and 10% fetal calf serum (Gibco). These cultures were maintained at 37°C in a humidified 95% O_2 /5% CO_2 atmosphere for 2–3 days. Transfection of the cells with recombinant rat $\alpha 1\beta 2\gamma 2$ or $\alpha 6\beta 2\gamma 2$ GABA_A receptors were carried out as mentioned above. For the identification of transfected cells 1 μg /plate of eGFP cDNA (Clontech) was added. Two days after transfection single coverslips containing HEK 293 cells were placed in a recording chamber mounted on the movable stage of a fluorescence microscope (Zeiss Axio-scope) and perfused with a defined saline solution containing (in mM): 140 NaCl, 5.4 KCl, 2 CaCl_2 , 2 MgSO_4 , 10 glucose, 5 sucrose, and 10 HEPES (free acid), pH adjusted to 7.35 with HCl. Transfected cells were identified by their eGFP fluorescence and ligand-mediated membrane currents of these cells were studied in the whole-cell configu-

ration of the patch-clamp technique (Hamill et al., 1981). Patch-clamp pipettes were pulled from hard borosilicate capillary glass (0.5 mm ID, 1.5 mm OD, Vitrex, Science Products, Hofheim, Germany) using a vertical puller (Narishige PP-83, Tokyo, Japan) in a two-stage process. The pipettes had an initial resistance of 3–5 M Ω when filled with a solution containing (in mM): 140 CsCl, 2 CaCl_2 , 2 MgCl_2 , 10 EGTA, 3.1 ATP (di-potassium salt), 0.4 GTP (tri-sodium salt), and 10 HEPES (sodium salt), pH 7.35. The values of the standard holding potential of -60 mV were not corrected for residual series resistance errors, being in the range of 5%. Using a fast Y-tube application system (Wulf Hevers, manuscript in preparation), test solutions containing GABA, 4-PIOL or GABA plus 4-PIOL were applied to the cells. The recombinant receptors were tested at their EC_{20} , EC_{50} , EC_{80} , and approximate EC_{100} values for GABA determined in preliminary experiments (data not shown) with 0.3, 3, 30, and 300 μM 4-PIOL. Responses of the cells were recorded by a patch-clamp amplifier (EPC-9, HEKA-Electronic, Lambrecht, Germany) in conjunction with a standard personal computer and the Pulse 9 software (HEKA). Whole cell currents were low-pass filtered by a eight-pole Bessel filter at 10 kHz before being digitized and recorded by the computer at a sampling rate of at least 1 kHz.

2.5. Statistics

Statistical significance of the differences from the overall average binding (to demonstrate brain regional heterogeneity in the actions of each drug tested), and between two population means was assessed using Student's *t*-test with InStat program (GraphPad Software, San Diego, CA). To assess regional differences in the agonist actions, Spearman rank correlations were determined between the percent values of GABA and other ligands.

3. Results

In rat brain sections, the GABA mimetics produced a widespread inhibition of [^{35}S]TBPS binding in agreement with the known agonistic effects on GABA_A receptors (Squires et al., 1983; Ticku and Ramanjaneyulu, 1984). All ligands used in the present investigation, except for 4-PIOL, diminished [^{35}S]TBPS binding at high concentration to less than 10% of the basal binding (data not shown). All brain areas appeared to be sensitive, but there was a clear quantitative regional variation. Table 1 depicts the brain regional inhibition of [^{35}S]TBPS binding by the full agonistic ligands at concentrations that roughly reduced the binding to half.

The profiles of muscimol and thiomuscimol were very similar to that of GABA. Though the profiles of THIP, taurine and β -alanine did not differ substantially, they produced a greater variability among the brain regions.

Table 1

Brain regional effects of various GABA_A agonists on picrotoxinin-sensitive [³⁵S]TBPS binding in rat brain sections

	Basal	GABA	Muscimol	Thiomusc.	THIP	Taurine	β-Alanine
<i>Olfactory areas</i>							
Olfactory bulb, external plexiform layer	165 ± 60	16 ± 7 ^a	14 ± 2 ^b	11 ± 2 ^b	21 ± 4 ^b	11 ± 3 ^b	13 ± 4 ^a
Islands of Calleja	331 ± 88 ^c	49 ± 13	32 ± 8	27 ± 3	78 ± 20	48 ± 12	33 ± 8
Olfactory tubercle	<u>55 ± 23^a</u>	39 ± 10	46 ± 10	43 ± 11	55 ± 8	56 ± 25	44 ± 11
<i>Cerebral cortex</i>							
Parietal cortex	182 ± 35	47 ± 7	30 ± 6	23 ± 5	68 ± 13	54 ± 8	33 ± 9
<i>Limbic regions</i>							
Anterior cingulate cortex	138 ± 42	31 ± 13 ^b	<u>16 ± 8^c</u>	<u>12 ± 1^b</u>	<u>23 ± 5^b</u>	<u>18 ± 8^a</u>	<u>16 ± 4^a</u>
Medial prefrontal cortex	105 ± 32	<u>21 ± 3^b</u>	<u>14 ± 3^a</u>	<u>15 ± 1^b</u>	25 ± 3	<u>16 ± 3^b</u>	21 ± 6
Subiculum	137 ± 28	52 ± 6	34 ± 4	31 ± 8	98 ± 26	54 ± 14	47 ± 11
Hippocampus	<u>82 ± 21^c</u>	45 ± 15	32 ± 1	24 ± 5	<u>48 ± 6^c</u>	43 ± 7	36 ± 8
Nucleus of horizontal limb of diagonal band	392 ± 114 ^c	46 ± 4	<u>24 ± 3^a</u>	<u>24 ± 3^c</u>	<u>96 ± 3^b</u>	47 ± 6	34 ± 4
Amygdala	100 ± 25	43 ± 9	31 ± 6	<u>20 ± 4^c</u>	<u>40 ± 5^a</u>	<u>38 ± 3^a</u>	<u>27 ± 4[*]</u>
Piriform cortex	115 ± 36	<u>37 ± 4^c</u>	29 ± 4	33 ± 4	64 ± 4	<u>29 ± 7^a</u>	39 ± 2 [*]
<i>Basal ganglia</i>							
Caudate-putamen	<u>81 ± 12^a</u>	52 ± 1 ^a	51 ± 7 ^c	43 ± 5 ^c	81 ± 6 ^c	78 ± 10 ^c	58 ± 3 ^b
Nucleus accumbens	<u>61 ± 21^a</u>	<u>33 ± 1^b</u>	45 ± 17	29 ± 3	<u>40 ± 8^a</u>	31 ± 17	32 ± 6
Globus pallidus	181 ± 24 ^c	71 ± 2 ^b	48 ± 5 ^a	50 ± 5 ^a	112 ± 12 ^a	78 ± 10 ^c	51 ± 4 ^a
<i>Thalamus</i>							
Thalamus	114 ± 15	43 ± 8	37 ± 4	33 ± 5	<u>52 ± 6^c</u>	47 ± 5	31 ± 3
Medial geniculate nucleus	150 ± 26	61 ± 5 ^a	29 ± 3	26 ± 5	71 ± 32	68 ± 7 ^c	32 ± 8
<i>Hypothalamus</i>							
Medial preoptic nucleus	<u>61 ± 19^a</u>	40 ± 8	25 ± 12	31 ± 15	<u>45 ± 5^a</u>	51 ± 21	<u>28 ± 1^a</u>
Paraventricular hypothalamic nucleus	<u>58 ± 23^a</u>	33 ± 11	34 ± 14	31 ± 7	<u>49 ± 9</u>	<u>35 ± 8</u>	37 ± 7
Medial hypothalamic nuclei	<u>73 ± 19^a</u>	54 ± 13	39 ± 12	27 ± 13	<u>46 ± 8^c</u>	62 ± 22	22 ± 8
<i>Midbrain</i>							
Superior colliculus, superficial gray layer	168 ± 35	58 ± 9	43 ± 10	27 ± 4	93 ± 20 ^c	81 ± 10 ^c	39 ± 12
Central gray	133 ± 26	55 ± 9	36 ± 5	28 ± 4	46 ± 12	57 ± 22	27 ± 5
Substantia nigra reticulata	208 ± 54	56 ± 9	31 ± 7	33 ± 6	89 ± 17	79 ± 2 ^b	35 ± 4
Red nucleus	128 ± 30	76 ± 12 ^c	54 ± 8 ^c	53 ± 15 ^c	119 ± 23 ^c	160 ± 8 ^b	64 ± 26
Inferior colliculus	240 ± 69	56 ± 5 ^c	31 ± 5	32 ± 3	104 ± 13 ^a	54 ± 6	48 ± 4 ^a
<i>Cerebellum</i>							
Cerebellar cortex, granule cell layer	130 ± 29	<u>27 ± 6^a</u>	29 ± 2	30 ± 4	<u>25 ± 5^b</u>	<u>40 ± 3^a</u>	<u>16 ± 2^b</u>
Cerebellar cortex, molecular layer	<u>83 ± 29^c</u>	<u>18 ± 1^b</u>	<u>27 ± 1^a</u>	27 ± 1	<u>31 ± 14^c</u>	<u>22 ± 2^b</u>	<u>21 ± 7^c</u>
Average	141	45	33	29	62	52	34

The concentrations of agonists tested were the following: (in μM) GABA, 3; muscimol, 1; thiomuscimol, 10; THIP, 30, and (in mM) taurine, 5; β-alanine, 1. The concentration of [³⁵S]TBPS was 6 nM. The data (mean ± SD, *n* = 4–5) for basal binding is given in nCi/g tissue equivalent or as percent of the basal for all agonists. Student's *t*-test was used to assess the significance of the differences from the corresponding average values (see footnotes a–c). The values significantly below the average values are underlined.

^a*P* < 0.01.

^b*P* < 0.001.

^c*P* < 0.05.

There were several α1 subunit-enriched regions, such as globus pallidus, substantia nigra, red nucleus and inferior colliculus (Wisden et al., 1992), where 30 μM THIP failed to reduce [³⁵S]TBPS binding at all, while in the rostral and caudal ends the binding was strongly inhibited. The regional effects of all these ligands highly correlated with those of GABA (Spearman correlation coefficient *r* > 0.48, *P* < 0.04), indicating that their actions share a few structural requirements.

In order to detect smaller differences in the action of presumed GABA site partial agonists, we studied the effects of GABA at 2 and 5 μM concentrations (Table 2). They reproduced the regional effects seen at 3 μM concentration (cf. Table 1), indicating that the averaged IC₅₀'s for GABA vary only by a factor of 2 to 3 between the brain regions. Piperidine-4-sulphonic acid strongly reduced [³⁵S]TBPS binding with regional variations rather similar to that observed for the full agonists. At a concentration of

Table 2

Effects of piperidine-4-sulphonate (P4S) and 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) on the rat brain regional binding of [35 S]TBPS to GABA_A receptors

	Basal	GABA, 2	GABA, 5	P4S, 10	G2 + P4S	G5 + P4S	4-PIOL, 300	G2 + 4-PIOL
<i>Olfactory areas</i>								
Olfactory bulb, external plexiform layer	178 ± 99	23 ± 7 ^a	11 ± 4 ^a	16 ± 4 ^b	8 ± 3 ^a	5 ± 2 ^a	45 ± 20 ^c	39 ± 16 ^a
Islands of Calleja	211 ± 43 ^c	64 ± 4	25 ± 4	68 ± 3 ^a	25 ± 4 ^c	14 ± 3	105 ± 8 ^c	96 ± 4
Olfactory tubercle	67 ± 14 ^a	56 ± 8	19 ± 9	37 ± 3 ^a	16 ± 3 ^c	10 ± 3	77 ± 20	79 ± 48
<i>Cerebral cortex</i>								
Parietal cortex	138 ± 20	76 ± 2 ^a	26 ± 4	59 ± 3	24 ± 2	15 ± 2	127 ± 22 ^c	113 ± 37
<i>Limbic regions</i>								
Anterior cingulate cortex	121 ± 24	57 ± 7 ^b	14 ± 4 ^a	34 ± 8 ^c	10 ± 5 ^c	6 ± 1 ^b	44 ± 5 ^a	36 ± 2 ^b
Medial prefrontal cortex	130 ± 13	40 ± 3 ^b	13 ± 3 ^a	22 ± 6 ^a	10 ± 5 ^c	7 ± 3 ^a	45 ± 4 ^b	39 ± 10 ^a
Subiculum	121 ± 5	68 ± 17	35 ± 7	62 ± 3 ^c	28 ± 1 ^c	15 ± 2	139 ± 35	167 ± 42 ^c
Hippocampus	122 ± 20	49 ± 6 ^c	21 ± 6	40 ± 4 ^a	20 ± 1 ^c	13 ± 3	75 ± 4 ^a	79 ± 8
N. of horizontal limb of diagonal band	251 ± 16 ^b	70 ± 1 ^b	28 ± 6	70 ± 6 ^c	29 ± 5	17 ± 3	103 ± 8	102 ± 5
Amygdala	133 ± 17	48 ± 11	28 ± 5	42 ± 1 ^b	18 ± 1 ^a	10 ± 3	62 ± 6 ^a	64 ± 4 ^a
Piriform cortex	115 ± 5 ^c	59 ± 7	21 ± 4	53 ± 10	18 ± 4	11 ± 1 ^a	81 ± 13	82 ± 7
<i>Basal ganglia</i>								
Caudate-putamen	77 ± 2 ^b	80 ± 4 ^a	29 ± 6	63 ± 4 ^c	28 ± 6	17 ± 2	89 ± 4	88 ± 8
Nucleus accumbens	87 ± 9 ^a	57 ± 7	18 ± 3 ^a	35 ± 4 ^a	10 ± 4 ^c	8 ± 2 ^a	47 ± 9 ^a	47 ± 11 ^a
Globus pallidus	131 ± 6	103 ± 11 ^a	42 ± 9 ^c	90 ± 11 ^a	45 ± 10 ^c	27 ± 6 ^c	134 ± 11 ^a	130 ± 9 ^a
<i>Thalamus</i>								
Thalamus	122 ± 15	61 ± 4	22 ± 5	53 ± 2	27 ± 4	17 ± 4	129 ± 10 ^a	120 ± 14 ^c
Medial geniculate nucleus	115 ± 12	70 ± 15	42 ± 6 ^c	72 ± 6 ^a	35 ± 2 ^a	21 ± 4 ^c	120 ± 10 ^c	122 ± 22
<i>Hypothalamus</i>								
Medial preoptic nucleus	90 ± 4 ^b	71 ± 12	24 ± 4	40 ± 11 ^b	12 ± 2 ^a	7 ± 2 ^a	59 ± 8 ^a	56 ± 16 ^c
Paraventricular hypothalamic nucleus	68 ± 4 ^b	56 ± 4 ^c	16 ± 3 ^a	33 ± 3	15 ± 1	9 ± 1 ^a	74 ± 23	78 ± 28
Medial hypothalamic nuclei	93 ± 26	65 ± 23	37 ± 5 ^c	48 ± 11	15 ± 10	13 ± 6	56 ± 14 ^c	64 ± 4 ^a
<i>Midbrain</i>								
Superior colliculus, superficial gray layer	132 ± 11	77 ± 16	40 ± 3 ^a	75 ± 8 ^c	38 ± 2 ^b	21 ± 3 ^c	121 ± 21	128 ± 28
Central gray	124 ± 13	76 ± 17	35 ± 7	72 ± 11 ^c	33 ± 6 ^c	17 ± 3	76 ± 19	85 ± 33
Substantia nigra reticulata	159 ± 27	69 ± 11	37 ± 6	72 ± 11 ^c	36 ± 4 ^a	21 ± 3 ^c	118 ± 14 ^c	121 ± 17 ^c
Red nucleus	102 ± 17 ^c	92 ± 12 ^c	49 ± 14	101 ± 16 ^a	54 ± 5 ^a	29 ± 7 ^c	150 ± 10 ^a	167 ± 39 ^c
Inferior colliculus	150 ± 21	79 ± 15	34 ± 9	77 ± 7 ^a	36 ± 6 ^c	19 ± 4	134 ± 18 ^c	140 ± 16 ^a
<i>Cerebellum</i>								
Cerebellar cortex, granule cell layer	209 ± 28 ^c	42 ± 9 ^c	20 ± 9	37 ± 6 ^c	10 ± 1 ^b	10 ± 3	119 ± 27	54 ± 10 ^a
Cerebellar cortex, molecular layer	110 ± 3 ^a	39 ± 6 ^a	16 ± 5 ^c	28 ± 4 ^a	7 ± 4 ^a	5 ± 1 ^b	77 ± 26	50 ± 13 ^a
Average	129	63	27	54	23	14	92	90

The concentrations of compounds are given in μM . The concentration of [35 S]TBPS was 6 nM. The data (mean $\pm$ SD, $n = 4-5$) for basal binding column is given in nCi/g tissue equivalent, and for other columns as percent of the basal binding. Student's t -test was used to assess the significance of the differences from the corresponding average values (see footnotes a–c). The values significantly below the average values are underlined.

^a $P < 0.01$.

^b $P < 0.001$.

^c $P < 0.05$.

10 μM , piperidine-4-sulphonic acid enhanced the action of both GABA concentrations (2 and 5 μM), and failed to antagonize the action of GABA. There was a strong correlation between the effects of piperidine-4-sulphonic acid and GABA ($r > 0.8$, $P < 0.0001$).

Another presumed partial agonist, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) reduced [35 S]TBPS binding in several brain regions (Fig. 1). In those regions where it alone decreased [35 S]TBPS binding, it failed to potentiate the GABA (2 μM) effect (Table 2, Fig. 1). Especially, the diencephalic and thalamic regions were not affected even

by the saturating concentration of 300 μM 4-PIOL. Still, 4-PIOL antagonized the effect of GABA in these brain regions. The cerebellum, especially the granule cell layer, was rather insensitive to 4-PIOL, since 4-PIOL affected neither the basal binding nor the GABA action, i.e., the cerebellar granule cell layer was the only region where the addition of GABA significantly ($P < 0.01$) affected the binding in the presence of 4-PIOL. Still, the correlation between the regional actions of GABA and 4-PIOL was high ($r = 0.8$, $P < 0.0001$), and improved with the coapplication of 4-PIOL and GABA ($r = 0.94$).

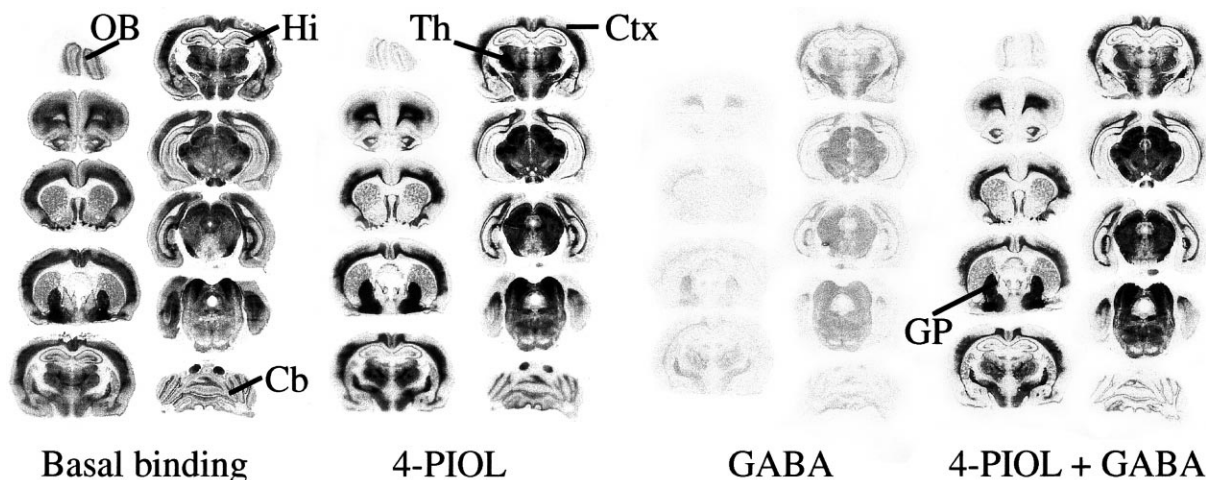


Fig. 1. Representative autoradiographs of [35 S]TBPS binding under basal conditions and in the presence of 300 μ M 5-(4-piperidyl)isoxazol-3-ol (4-PIOL), 2 μ M GABA and 300 μ M 4-PIOL plus 2 μ M GABA. The concentration of [35 S]TBPS was 6 nM. The images demonstrate increases and decreases in the binding in selected brain regions by 4-PIOL as compared to the basal binding. GABA strongly decreases the binding, but its effect is counteracted by 4-PIOL in most brain regions, except in the cerebellum. OB, olfactory bulb; Hi, hippocampus; Th, thalamus; Ctx, cerebral cortex; Cb, cerebellum; GP, globus pallidus.

To better model the cerebellar GABA_A receptors, recombinant $\alpha 1\beta 2\gamma 2$ receptors as the main GABA_A receptor subtype and $\alpha 6\beta 2\gamma 2$ receptors as the cerebellum-specific subunit combination (Lüddens et al., 1990) were used to test the effects of 4-PIOL on [35 S]TBPS binding in the absence and presence of GABA. The 4-PIOL effects on [35 S]TBPS binding clearly differentiated the subtypes, as 4-PIOL increased the binding in $\alpha 1\beta 2\gamma 2$ receptors independently of GABA (Fig. 2A) and affected the $\alpha 6\beta 2\gamma 2$ receptors only at the highest 4-PIOL concentration (300 μ M) by weakly antagonizing the GABA effect (Fig. 2B). Electrophysiological experiments on the two GABA_A receptor subtypes complemented the binding studies, and revealed remarkable differences between the recombinant GABA_A receptors. For the $\alpha 1\beta 2\gamma 2$ combination, 4-PIOL was dose-dependently agonistic in most cells tested (18 of 27), although at 300 μ M it increased the membrane current amplitudes maximally to only 5% of GABA-induced currents (Fig. 3A). In cells transfected with $\alpha 6\beta 2\gamma 2$ receptors, 4-PIOL failed to evoke any current even at the concentration of 300 μ M (Fig. 3C). To determine the basis of the partial agonism of 4-PIOL in $\alpha 1\beta 2\gamma 2$ receptors, solutions containing GABA at the predetermined EC₂₀, EC₅₀, EC₈₀ and approximate EC₁₀₀ concentrations were co-applied with 0.3, 3, 30 and 300 μ M 4-PIOL. GABA-induced whole-cell currents were reduced to 20% by 300 μ M 4-PIOL at the EC₂₀, EC₅₀ and EC₈₀ (Fig. 3B). The dose-response curves for 4-PIOL at these GABA concentrations were not significantly different, whereas it lost its efficacy at 100 μ M GABA (EC₁₀₀). Contrary to the low-potency antagonistic effects of 4-PIOL on the cerebellar granule cell and recombinant $\alpha 6\beta 2\gamma 2$ receptors in the binding assays, the electrophysiological

experiments suggested similar antagonistic potencies of 4-PIOL both in $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2\gamma 2$ receptors in spite of their divergent agonistic properties. At all GABA concentrations, except for the approximate EC₁₀₀ concentra-

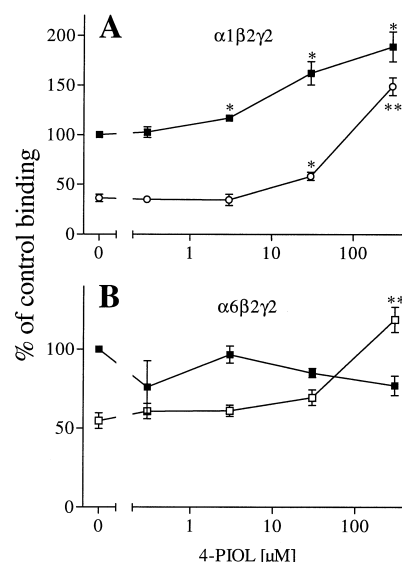


Fig. 2. Effect of increasing concentrations of 5-(4-piperidyl)isoxazol-3-ol (4-PIOL) on [35 S]TBPS binding in recombinant $\alpha 1\beta 2\gamma 2$ (A) and $\alpha 6\beta 2\gamma 2$ (B) GABA_A receptors. The concentration of [35 S]TBPS was 6 nM. The results (% of control binding) are means $\pm$ SEM for three independent determinations. Symbols indicate GABA concentrations: no GABA ($\blacksquare$), 3 μ M and 1 μ M ($\square$) for A and B, respectively. The control binding values for $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors were 544 ± 342 and 50 ± 18 fmol/mg protein, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for the statistical significance of the difference from the corresponding value without 4-PIOL (paired t -test).

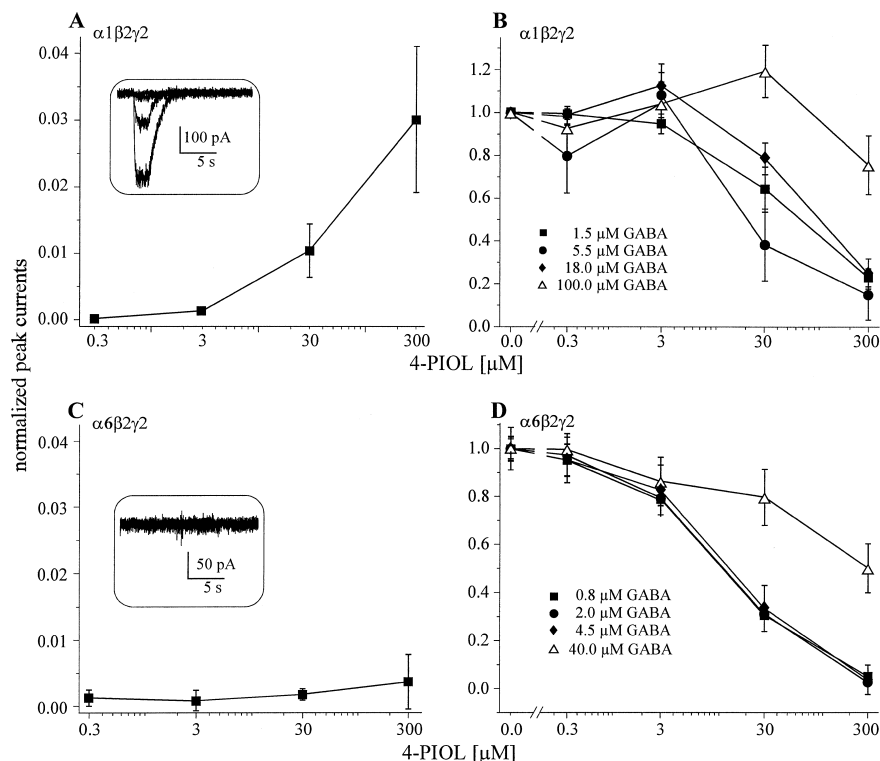


Fig. 3. Whole-cell recordings of HEK 293 cells expressing recombinant rat $\alpha 1\beta 2\gamma 2$ (A,B) and $\alpha 6\beta 2\gamma 2$ receptors (C,D). Currents were normalized to the maximal GABA-induced response. Insets in A and C are original recordings of currents evoked by 4-PIOL. In B and D, GABA was applied at concentrations of EC₂₀ (■), EC₅₀ (●), EC₈₀ (◆), and EC₁₀₀ (△) as shown for $\alpha 1$ and $\alpha 6$ subunit-containing receptors. Different concentrations of 4-PIOL were co-applied with GABA. Error bars indicate the standard derivation ($\pm$ SD) for five to six cells.

tion, currents were strongly diminished to 5–10% by 300 μ M 4-PIOL (Fig. 3D).

4. Discussion

In the present study, we demonstrate by using the functional [³⁵S]TBPS ligand autoradiography on rat brain sections that various endogenous agonistic compounds acting on the GABA recognition site fail to display any dramatic brain regional differences. This is in agreement with the data of Bureau and Olsen (1993), who found that the brain regional IC₅₀ values for several GABA site agonists vary only within one order of magnitude in displacing [³H]muscimol binding from GABA_A receptors. However, we provide now evidence that the synthetic weak partial agonist 4-PIOL shows more pronounced brain regional differences. Functional analysis with electrophysiological recordings on recombinant rat GABA_A receptors suggests that the GABA sites or their coupling to the ion channels are heterogeneous with respect to this compound.

4.1. Similar brain regional efficacies of GABA, taurine, β -alanine and muscimol

We found that the regional efficacy profiles of the endogenous compounds taurine and β -alanine were similar

to that of GABA, although taurine had previously been suggested to preferentially act on $\beta 2$ subunit-rich areas on the basis of [³H]muscimol autoradiographic images (Bureau and Olsen, 1991). Also the GABA_A receptor agonists muscimol and thiomuscimol faithfully followed the profile of GABA, indicating an interaction with native GABA_A receptor subtypes similar to that of GABA. GABA, β -alanine and taurine widely differ in their potency to affect [³⁵S]TBPS binding, GABA being about 300 and 1000 times more potent than β -alanine and taurine, respectively. Therefore, the agonistic actions of β -alanine and taurine on their own on GABA_A receptors in vivo are likely to be limited.

4.2. THIP and piperidine-4-sulphonic acid are agonists in [³⁵S]TBPS binding, with slightly more variation in their efficacies on different brain regions

The synthetic agonist THIP differed from the endogenous agonists mentioned above in that its efficacy varied more between brain regions, suggesting that its interaction may partially depend on the receptor subtype. Our finding corroborate recent observations on recombinant GABA_A receptors expressed in *Xenopus laevis* oocytes (Ebert et al., 1997), where the efficacy of THIP in electrophysiological studies was the same as that of GABA, muscimol and

thiomuscimol in $\alpha 2$, $\alpha 5$ and $\alpha 6$ subunit-containing receptors, but clearly lower in $\alpha 1$ and $\alpha 3$ containing receptors.

Piperidine-4-sulphonic acid has been previously described as a partial agonist (Wong and Iversen, 1985). However, in the [35 S]TBPS binding assay employed here, piperidine-4-sulphonic acid acted as a full agonist. It enhanced the action of low and high GABA concentrations, and when applied alone at higher concentrations it prevented most of the [35 S]TBPS binding. Since piperidine-4-sulphonic acid strongly inhibited [35 S]TBPS binding in all brain regions, our assay argues for a full agonistic action of piperidine-4-sulphonic acid in most or all native receptors, in contrast to Ebert et al. (1994), who found functional evidence for partial agonism of piperidine-4-sulphonic acid in recombinant $\alpha 1$ subunit-containing receptors.

4.3. 4-PIOL is a weak agonist / antagonist with receptor subtype selectivity

Another putative partial agonist, 4-PIOL, had limited efficacy in the [35 S]TBPS binding assay, consistent with partial agonism (Krogsgaard-Larsen et al., 1994), but it failed to add to the effect of a low GABA concentration even in those brain areas where major differences were seen between 2 and 5 μ M GABA. Actually, in most brain regions it completely inhibited the action of GABA. Therefore, 4-PIOL can be considered a partial agonist. 4-PIOL was without effect in several brain regions, e.g., in the cerebellar granule cell layer, where it lacked an effect both in the presence and absence of GABA. However, 4-PIOL significantly elevated [35 S]TBPS binding above basal levels in many $\alpha 1$ subunit-containing brain regions, which is characteristic for this receptor subtype at low GABA concentrations (Korpi and L  ddens, 1993) and consistent with low efficacy agonism. Therefore, the failure to show any effect in a brain region rich in $\alpha 6$ subunit-containing receptors could be due to a masking by the summation of the enhancing and decreasing effects on $\alpha 1$ and $\alpha 6$ subunit-containing receptors, respectively. This hypothesis is substantiated by our findings on recombinant receptors, as $\alpha 1\beta 2\gamma 2$ receptors bound more [35 S]TBPS in the presence than in the absence of 4-PIOL, while no increase of binding was observed in $\alpha 6\beta 2\gamma 2$ receptors under the same incubation conditions, i.e., $149 \pm 15\%$ and $95 \pm 15\%$ of control values for $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors, respectively, in the presence of 3 μ M GABA plus 300 μ M 4-PIOL.

In line with these findings, our electrophysiological data revealed clear differences between recombinant receptor subtypes. In the absence of GABA, 4-PIOL acted as a weak agonist in $\alpha 1\beta 2\gamma 2$ receptors by eliciting currents up to 5% of the maximal GABA response, but it failed to evoke any current in $\alpha 6\beta 2\gamma 2$ receptors. Interestingly, Kristiansen et al. (1991) observed that 4-PIOL is unable to produce bursts of GABA_A receptor channel openings of

long duration in embryonic olfactory bulb neurons, indicating that 4-PIOL fails to induce a proper conducting state of the chloride channel. In our hands, the compound reduced current amplitudes in a dose-dependent manner in $\alpha 1$ - and $\alpha 6$ -containing receptor isoforms independent of the GABA concentrations used, except for the one approaching the EC₁₀₀, thus exhibiting characteristics of an antagonist. However, these data cannot clearly differentiate between competitive and noncompetitive forms of inhibition. The partial agonism efficacy seen in $\alpha 1$ subunit-deficient brain regions contrasts with findings that the thiol derivative of 4-PIOL acts as a pure antagonist in human recombinant GABA_A receptors assembled from $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$ or $\alpha 6$ subunits with a β and the $\gamma 2$ subunit (Ebert et al., 1997), which may, however, be due to the thiol function.

4.4. Receptor subunit correlations with efficacies of GABA site ligands

The second purpose of the present study was to obtain a correlation of the action of GABA site agonists with the brain region-specific expression of the GABA_A receptor subunits. $\alpha 2$ subunit-enriched brain regions were slightly more sensitive to the agonists than most $\alpha 1$ subunit-containing regions. However, there was as much variance in the effects of all compounds among $\alpha 1$ subunit-enriched regions as in regions abundantly expressing other α subunits, indicating that there is no simple α subunit-based correlation with the effects of GABA agonists. This can be clearly demonstrated in the cerebellar granule cell layer with its unique population of $\alpha 6$ subunit-containing receptors, which displays about 30-fold higher GABA sensitivity when compared to $\alpha 1$ subunit-containing receptors (Korpi and L  ddens, 1993). Even when employing an improved preincubation strategy to remove excess endogenous GABA and other agonists, the success of which can be evidenced by an increased [35 S]TBPS binding in that brain region (see M  kel   et al., 1997), the agonist effects were only slightly more pronounced in the cerebellar granule cell layer than on average. Furthermore, in the olfactory bulb and cerebellar molecular layer, both enriched in $\alpha 1$ but devoid of $\alpha 6$ subunit-containing receptors, GABA was as efficient as in the granule cell layer.

The assay systems that were used to determine GABA_A receptor sensitivities gave slightly different results. For example, the autoradiographic method failed to show any antagonism of the cerebellar granule cell layer effect of GABA by 4-PIOL, whereas in recombinant $\alpha 1$ and $\alpha 6$ subunit-containing receptors 4-PIOL was very efficient as an antagonist. This has to be kept in mind, since both methods complement each other, the main difference being that the autoradiography detects several receptor subtypes in each brain region, whereas the recombinant system usually expresses only a single one.

In conclusion, although most of the GABA site ligands had very similar brain regional profiles, the weak partial

agonist/antagonist properties of 4-PIOL showed subtype-specific characteristics that might make it a useful compound to compare the structure–function relationships and mechanisms of the coupling between GABA and ionophore sites in both various native and recombinant GABA_A receptor subtypes and chimeric and mutant receptors.

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